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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) <p>This research aims to understand the mechanism by which the catenin p120 regulates the cell adhesion molecule E-cadherin, a protein whose function is critical to inhibiting metastasis of human tumors. My hypothesis is that uncoupling p120 from E-cadherin by mutation of the juxtamembrane domain will result in an E-cadherin molecule that is defective for the function contributed by p120. In this reporting period, I have mutated the p120 binding site on E-cadherin and generated minimally altered E-cadherin proteins that are selectively unable to bind p120. Expression of the p120-uncoupled cadherin proteins in the E-cadherin deficient human breast cancer cell line MDA231 eliminates the ability of E-cadherin to induce tightly organized epithelial colonies. The cells form poor junctions and are not able to mediate strong cell-cell adhesion. We are planning to analyze the metastatic potential of these cells in mice. These results significantly clarify the role of p120 in cadherin function and could ultimately lead to rational therapeutic approaches to inhibit metastasis of breast cancer.</p>				
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INTRODUCTION

Cell-cell adhesion is a fundamental property of epithelial cells which is lost during the transition from solid to metastatic tumors. E-cadherin is the major cell adhesion molecule involved in this process, and evidence indicates that defects not only in the cadherin itself, but also in its cytoplasmic binding partners can affect the transition to metastasis. We aim to better understand this process by studying the interaction of E-cadherin with p120, a src substrate and cadherin binding partner discovered in our lab. To dissect the influence of p120 on E-cadherin function, we proposed to uncouple p120 from E-cadherin *in vivo* and examine effects on the cadherin related to cell adhesion and invasion. These experiments seek to understand at the mechanistic level, the epithelial to mesenchymal transition which occurs as a loss of cadherin function, and may suggest targets for inhibiting progression to metastasis.

BODY

Training: During the past year, our lab has joined the new Cancer Biology Department, which was formed last year under the leadership of Dr. Lynn Matrisian. I have helped to initiate a Cancer Biology Student Association which organizes informational activities on various aspects of cancer research including new techniques, speaking skills, and career planning. Last summer, I attended the "Pathobiology of Cancer" workshop sponsored by the American Association of Cancer Research. The workshop gave me an intense exposure to the histology of cancer, as well as a deeper understanding of the molecular mechanisms involved in cancer progression. This summer I attended an excellent Gordon Conference on "Cell Contact and Adhesion."

Research: The statement of work for this grant is included below for reference. We have completed Tasks 1 and 2, and parts of Task 3, and these results are published in the Journal of Cell Biology¹ (see Appendix). For Task 1, I subcloned the E-cadherin mutants 761AAA and 764AAA (which are uncoupled from p120) into the pLK expression vector. These mutants were then transfected into MDA231 cells to obtain stable cell lines. Simultaneously, A431D cells were also transfected with the same constructs, and experiments were carried out in both cell lines. Cells were screened by immunofluorescence and western blotting for E-cadherin, p120, and several other catenins (α , β , and γ). These experiments indicated that unlike α and β catenin, which are degraded if not bound to cadherins, p120 was stable and accumulated in the cytoplasm when uncoupled from cadherins. For unknown reasons, I was not able to achieve stable clones of the 764AAA mutant in MDA231 cells.

In Task 2, I characterized the cell lines obtained. I performed co-immunoprecipitations of p120 with the 761AAA and 764AAA E-cadherin mutants which show that p120 does not bind to these E-cadherin mutants. In contrast, α and β catenin bind very well to both wild-type and mutant E-cadherin, demonstrating that the interaction with p120 was selectively abrogated. Subcellular fractionation was performed and revealed, in contrast to previous reports, that over 90% of p120 associates with E-cadherin. Last, treatment of cell lysates with lambda phosphatase confirmed that p120 is phosphorylated when bound to cadherins at the cell membrane, but not phosphorylated while cytoplasmic. The use of p-tyr antibodies showed no tyrosine phosphorylation of p120. By phospho-amino acid

analysis, we determined that phosphorylation under these conditions was almost exclusively on serine. This modification may prove to be crucial to p120 function and others in the lab are currently mapping the p120 serine phosphorylation sites.

Task 3 was approached by performing assays for cadherin function. An initial observation from the cell lines created was that cells containing mutant E-cadherin appeared to have loose junctions and were unable to form tight colonies on tissue culture plates. Staining of the actin cytoskeleton revealed disorganized actin filaments in cells containing mutant E-cadherin. Circumferential actin cables which encircle cell colonies and result in compaction were absent from mutant cells, even though cadherins were able to concentrate at junctions. These observations suggested a defect in cadherin clustering due to the lack of p120 binding, and this may partially be explained by our observations that p120 inhibits the activity of RhoA². We therefore proceeded with aggregation assays, and discovered that while wild-type cadherin promoted strong cell adhesion, mutant cadherin supported only weak adhesion, in which cells could easily be dissociated by pipetting. Motility experiments revealed no significant differences between wild-type and mutant cells, although difficulties in these experiments were encountered because the cells began to rapidly lose cadherin expression. These experiments will be repeated when homogenously expressing cell lines are obtained (see Task 4). Unfortunately, neither cell line grew in soft agar in our experiments, and these experiments are currently being optimized. In addition, we are using a dimerized E-cadherin extracellular domain (obtained from Barry Gumbiner) to assess differences in clustering ability in wild-type versus mutant cells.

We are in the process of beginning Task 4 of this grant. A problem that we have encountered in this line of experiments is that the "stable" cell lines we have generated often lose expression of the cadherin, which complicates interpretation of experiments that take place over long periods of time. We have worked around this problem by using cells only at low passage numbers. However, to generate dependable data in animal studies, we need to improve the stability of these cell lines. Therefore, we have obtained a retroviral vector (LZRS-pBMN-GFP) from Garry Nolan at Stanford University. Unlike the cells expressing E-cadherin from the dexamethasone-inducible pLK promoter, this vector has yielded extremely stable transfected cell lines. The vector is unique in that after the gene of interest (E-cadherin in this case), there is an Internal Ribosomal Entry Site (IRES) followed by the Green Fluorescent Protein (GFP) gene. This allows both genes to be made on one transcript, with the outcome being that GFP expression and E-cadherin expression correspond perfectly in cells, providing an easy readout for analyzing expression in live cells. We anticipate that the GFP expression will also be useful for tracking tumor cells in the mice. Alternatively, we are planning to insert an antibiotic resistance gene in place of the GFP gene to facilitate selection of expressing cells. Cell lines will be made using the vector alone and wild-type or mutant E-cadherin in MDA-231 cells as planned previously, and these will be injected into mice to analyze p120's contribution to metastasis. These experiments will help to reveal the metastatic contribution of p120 uncoupling from E-cadherin.

STATEMENT OF WORK

Task 1: To generate the MDA231 model system for uncoupling E-cadherin and p120 function (months 1-12).

- A. Subclone E-cadherin AAA mutants from yeast pGad vector to pLKpac1 expression vector.
- B. Transfect E-cadherin mutants into MDA231 cells and establish stable cell lines expressing these mutants.
- C. Screen cell lines for E-cadherin expression by immunofluorescence, and western blotting.

Task 2: To characterize stably transfected cell lines and identify minimal E-cadherin JMD mutants that effectively uncouple E-cadherin from p120 (months 6-18).

- A. Characterize binding of wild-type and mutant E-cadherin to p120 and α - and β -catenin by co-immunoprecipitation.
- B. Determine final mapping of p120 binding site on E-cadherin's JMD.
- C. Perform subcellular fractionation of transfected cells to address p120/E-cadherin stoichiometry.
- D. Phosphatase-treat p120 immunoprecipitates and blot for p-tyr, ser and thr to assess p120 phosphorylation state when bound to/uncoupled from E-cadherin.

Task 3: To determine the impact of uncoupling p120 and E-cadherin on adhesion, motility, and invasion by performing functional assays *in vitro*. (months 12-36).

- A. Perform aggregation assays using established protocols for cadherin function.
- B. Examine motility using an *in vitro* wound healing assay.
- C. Perform invasion assays in soft agar.

Task 4: To analyze the effects of uncoupling p120 and E-cadherin on metastasis utilizing a mouse model for metastasis of breast cancer to bone (months 24-36)

- A. Inject 24 female Balb/c-nu/nu (nude) mice with MDA231 cells transfected with vector alone, wild-type E-cadherin, or p120-uncoupled E-cadherin mutants.
- B. Assess tumor size (radiographically) and number in animals.
- C. Test ability of tumors to metastasize to bone using histological examination.
- D. Measure growth rate and tumorigenicity of cells by injection into the mammary fat pad of nude mice.

KEY RESEARCH ACCOMPLISHMENTS

- Uncoupled p120 from E-cadherin through a small three amino substitution in the conserved juxtamembrane domain of E-cadherin and made cell lines expressing these mutants.
- Demonstrated that p120 localizes to the cytoplasm in cadherin deficient or cadherin-uncoupled cells, where it is stable but unable to be serine phosphorylated.
- Established that cadherin expression is necessary and sufficient for p120 localization to cell junctions.
- Showed that p120 is involved in adhesive strengthening, supporting a role for p120 in cadherin clustering.

REPORTABLE OUTCOMES

Published our findings in the Journal of Cell Biology:

Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK and Reynolds AB. (2000) Selective Uncoupling of p120ctn from E-cadherin Disrupts Strong Adhesion. *Journal of Cell Biology*, **148**:189-202.

Developed stable cell lines in both MDA231 cells and A431D cells which contain either wild-type or p120-uncoupled E-cadherin.

CONCLUSIONS

As described above, we have made significant progress in this toward understanding p120 and its effects on E-cadherin and cadherin-mediated cell adhesion. We have established that the p120/E-cadherin interaction in epithelial cells occurs at high stoichiometry. Interestingly, unlike the other catenins, p120 can accumulate to high levels in the cytoplasm, where it may be involved in signaling pathways. A key finding is that the binding of p120 to E-cadherin is crucial for E-cadherin's ability to mediate strong cell-cell adhesion. We are currently investigating the mechanism of this regulation, as well as generating cell lines which can be used in metastasis studies in mice. These observations have proved critical in our understanding of p120's role in cell adhesion and signaling, and have lead to a better insight into the factors influencing cadherin-mediated cell adhesion.

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APPENDICES

Journal of Cell Biology manuscript
Curriculum Vitae, Molly Thoreson

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EDUCATION

1996-present	<i>Vanderbilt University</i>	Nashville TN
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EXPERIENCE

1996	Research Assistant with Dr. Randy Blakely, Department of Pharmacology, Vanderbilt University
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1993-1996	Laboratory Teaching Assistant, Biology and Genetics Courses, Texas Lutheran

AWARDS AND FUNDING SOURCES

2000 - present	Pre-doctoral Traineeship Grant, U.S. Department of Defense Breast Cancer Research Program (3 years, total award: \$64,347.)
1999	American Association of Cancer Research Science Education Award for Students
1997-00	Trainee, Army Breast Cancer Training Grant
1995	National Science Foundation-Sponsored Competitive SURF (Summer Undergraduate Research Fellowship) Award

PUBLICATIONS

Anastasiadis PZ, Moon SY, Thoreson MA, Mariner DJ, Crawford HC, Zheng Y, and Reynolds AB. (2000) Inhibition of RhoA by p120 catenin. *Nature Cell Biology*, 2:637-644.

Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK and Reynolds AB. (2000) Selective Uncoupling of p120ctn from E-cadherin Disrupts Strong Adhesion. *Journal of Cell Biology*, 148:189-202.

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SELECTED POSTERS AND PRESENTATIONS

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Thoreson MA. "Making and Breaking Junctions in Metastatic Cells: The Role of p120 and E-cadherin" 1999. Breast Cancer Research Seminar, Vanderbilt Cancer Center, Vanderbilt University, Nashville TN.

Thoreson MA, Hummingbird DK and Reynolds AB. p120ctn Binds the Conserved Juxtamembrane Domain of E-cadherin. Graduate Student Research Day, 1998. Vanderbilt University, Nashville TN.

Thoreson MA, Hummingbird DK and Reynolds AB. Role of p120ctn Interaction with E-cadherin. 37th Annual Meeting of the American Society of Cell Biology, 1997. Washington, DC.

Selective Uncoupling of p120^{ctn} from E-cadherin Disrupts Strong Adhesion

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Abstract. p120^{ctn} is a catenin whose direct binding to the juxtamembrane domain of classical cadherins suggests a role in regulating cell-cell adhesion. The juxtamembrane domain has been implicated in a variety of roles including cadherin clustering, cell motility, and neuronal outgrowth, raising the possibility that p120 mediates these activities. We have generated minimal mutations in this region that uncouple the E-cadherin-p120 interaction, but do not affect interactions with other catenins. By stable transfection into E-cadherin-deficient cell lines, we show that cadherins are both necessary and sufficient for recruitment of p120 to junctions. Detergent-free subcellular fractionation studies indicated that, in contrast to previous reports, the stoichiometry of the interaction is extremely high. Unlike α - and β -catenins, p120 was metabolically stable in cadherin-deficient cells, and was present at high levels in the cytoplasm. Analysis of cells expressing E-cadherin

mutant constructs indicated that p120 is required for the E-cadherin-mediated transition from weak to strong adhesion. In aggregation assays, cells expressing p120-uncoupled E-cadherin formed only weak cell aggregates, which immediately dispersed into single cells upon pipetting. As an apparent consequence, the actin cytoskeleton failed to insert properly into peripheral E-cadherin plaques, resulting in the inability to form a continuous circumferential ring around cell colonies. Our data suggest that p120 directly or indirectly regulates the E-cadherin-mediated transition to tight cell-cell adhesion, possibly blocking subsequent events necessary for reorganization of the actin cytoskeleton and compaction.

Key words: metastasis • catenin • compaction • clustering • adherens junction

Introduction

p120 catenin (p120^{ctn} or p120)¹ is an armadillo repeat protein (Reynolds et al., 1992; Peifer et al., 1994) whose direct binding to classical cadherins suggests a role in regulating cell-cell adhesion (Reynolds et al., 1994; Daniel and Reynolds, 1995; Shibamoto et al., 1995; Staddon et al., 1995). Originally discovered as a substrate of the Src oncoprotein (Reynolds et al., 1989), p120 is phosphorylated on both tyrosine and serine residues in response to a variety of growth factors such as EGF, PDGF, and CSF-1 (Downing and Reynolds, 1991; Kanner et al., 1991). In addition to its main localization in adherens junctions, p120 has been detected in the nucleus, particularly in metastatic cells that have lost cadherin expression (van Hengel et al., 1999), and it interacts directly with Kaiso, a novel transcription

factor of unknown function (Daniel and Reynolds, 1999). Together, these data suggest an interplay between signaling and adhesion systems that is likely to be important for regulating both cell proliferation and morphology.

Cadherins comprise a family of transmembrane cell surface glycoproteins that mediate calcium-dependent homotypic cell-cell adhesion through their extracellular domains, and regulate a variety of biological processes including development, morphogenesis, and tumor metastasis (for review see Takeichi, 1995; Yap et al., 1997). Cadherin extracellular domains bind one another on adjacent cells, whereas the intracellular domains anchor the cadherin complex to the actin cytoskeleton via proteins called catenins. β -Catenin and plakoglobin act as a bridge connecting E-cadherin to α -catenin (Aberle et al., 1994; Hulsken et al., 1994; Funayama et al., 1995; Jou et al., 1995; Rimm et al., 1995), a vinculin-like protein that is thought to associate with actin filaments either by direct interaction, or indirectly through association with α -actinin (Herrenknecht et al., 1991; Nagafuchi et al., 1991; Knudsen et al., 1995; Nieset et al., 1997). Unlike β -catenin

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¹Abbreviations used in this paper: APC, adenomatous polyposis coli; Dex, dexamethasone; JMD, juxtamembrane domain; MDA231, MDA-MB-231; p120, p120 catenin; RT, room temperature; wt, wild-type.

and plakoglobin, p120 does not bind α -catenin or the tumor suppressor adenomatous polyposis coli (APC), indicating that its role is fundamentally different from that of the other catenins (Daniel and Reynolds, 1995).

Multiple lines of evidence indicate that dysfunction of E-cadherin complexes promotes tumor metastasis (for review see Yap, 1998). E-cadherin expression is frequently downregulated or abrogated in metastatic carcinomas (for review see Birchmeier and Behrens, 1994), and ectopic expression in E-cadherin-deficient carcinoma cell lines strongly reduces their invasive phenotype in vitro (Frixen et al., 1991; Vleminckx et al., 1991) and in vivo (Perl et al., 1998). Although E-cadherin loss is generally an epigenetic event that occurs late in tumorigenesis, mutations or deletions in the gene itself are common in lobular carcinoma of the breast (Berx et al., 1995) and in gastric tumors (Oka et al., 1992), suggesting that in some instances E-cadherin loss may be an early, if not causal event. Although less frequently observed, defects in α - and β -catenins have also been reported and cause adhesive dysfunction resembling the consequences of E-cadherin loss (Hirano et al., 1992; Morton et al., 1993; Oyama et al., 1994; Kawanishi et al., 1995). p120 has also been reported to be absent, downregulated, or mislocalized in many tumors (Dillon et al., 1998; Gold et al., 1998; Shimazui et al., 1996; Skoudy et al., 1996; Valizadeh et al., 1997; Syrigos et al., 1998), and in some cases these changes correlate with poor prognosis.

In addition, it is clear that β -catenin has signaling roles important in development and cancer that may be largely independent of its role in cadherin complexes (for review see Peifer, 1995). Inactivating mutations in APC, or direct activating mutations in β -catenin, cause the accumulation of β -catenin in the cytoplasm and nucleus, leading to constitutive signaling mediated through interaction with the transcription factor Lef1/TCF (Munemitsu et al., 1995; Behrens et al., 1996; Molenaar et al., 1996; Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). Interestingly, p120 also interacts with a transcription factor, the novel POZ/zinc finger protein Kaiso, which is unrelated to Lef1/TCF (Daniel and Reynolds, 1999). p120 has been reported in the nucleus under certain conditions, and accumulates in the nucleus upon leptomycin treatment (van Hengel et al., 1999), suggesting that like β -catenin, it has roles in both cell-cell junctions and in the nucleus.

Separate, highly conserved binding sites for β -catenin and p120 on the cadherin cytoplasmic domain suggest crucial and indispensable roles for both proteins in cadherin function. p120 binds to the cadherin juxtamembrane domain (Finnemann et al., 1997; Navarro et al., 1998; Yap et al., 1998), whereas β -catenin and/or plakoglobin bind a distinct site on the COOH terminus of the cadherin molecule (the catenin binding domain) (Nagafuchi and Takeichi, 1988; Nagafuchi and Takeichi, 1989; Ozawa et al., 1990; Stappert and Kemler, 1994). Deletion studies reveal roles for the cadherin juxtamembrane domain in several activities, implicating p120 as the likely downstream effector. In *Xenopus*, the juxtamembrane domain is crucial for cell-cell adhesion during embryogenesis (Kintner, 1992). The juxtamembrane domain also appears to regulate neuronal outgrowth activity mediated by N-cadherin (Riehl et al., 1996), the selective exclusion of N-cadherin from junctions by VE-cadherin in endothelial cells (Navarro et al., 1998),

and the suppression of cell motility by E-cadherin (Chen et al., 1997). Ectopic expression experiments in CHO cells implicate the juxtamembrane domain in cadherin clustering and adhesive strengthening (Yap et al., 1998). However, work by Ozawa and Kemler (1998) suggests that the juxtamembrane domain negatively regulates adhesion activity and that p120 may be directly responsible for this effect (Ozawa and Kemler, 1998; Ohkubo and Ozawa, 1999). These data are consistent with the results of Aono et al. (1999), showing that p120 can function as an inhibitory regulator of adhesion in Colo205 cells. The conflicting data might be due in part to differences between various cell lines and systems. Alternatively, the use of a variety of relatively large deletions to disrupt the juxtamembrane domain might introduce conformational changes or uncouple interactions with other proteins in addition to p120.

To clarify the role of p120 in cadherin function, we have fine-mapped the p120 binding site in the E-cadherin juxtamembrane domain using three amino acid alanine substitutions, and established minimal E-cadherin mutants designed to limit conformational effects while selectively eliminating the interaction with p120. Expression of wild-type (wt) and p120-uncoupled versions of E-cadherin in cadherin-deficient cell lines revealed clearly that cadherin expression is both necessary and sufficient for recruitment of p120 to cell-cell junctions. Moreover, in contrast to previous work, we found that over 90% of the cellular p120 was cadherin associated, suggesting a stoichiometric interaction. Unlike α - and β -catenins, p120 was metabolically stable in cadherin-deficient cells and accumulated to high levels in the cytoplasm. Given the apparent lack of any mechanism to degrade cytoplasmic p120, one possibility is that association with E-cadherin is an important means of restricting p120 access to the nucleus. Cadherin association was also required for phosphorylation of p120, whereas cytoplasmic p120 was almost completely unphosphorylated. Interestingly, the p120-uncoupled E-cadherin mutants were deficient in mediating cell-cell compaction. Subcellular localization of actin revealed defects in the continuity of the circumferential ring that normally links epithelial cells into continuous compact colonies. In aggregation assays, the p120-uncoupled E-cadherin failed to mediate tight adhesion, indicating that p120 is required for the E-cadherin-regulated transition from loose to tight adhesion.

Materials and Methods

Yeast Two-Hybrid Assay and Generation of Mammalian Expression Plasmids

As previously described, a cDNA clone encoding the cytoplasmic domain of human E-cadherin was generated by PCR, sequenced, and subcloned into the yeast two-hybrid vector pGAD to generate pGAD-EcadCD (Daniel and Reynolds, 1995). Site-directed mutagenesis was carried out using the Chameleon mutagenesis kit (Stratagene) to introduce point mutations or triple alanine substitutions throughout the juxtamembrane domain. The resulting mutant DNA was sequenced to verify the absence of secondary mutations, and then assayed in the yeast two-hybrid system, as described previously in Daniel and Reynolds (1995), for direct interaction with p120. The mutated human E-cadherin cytoplasmic domains were substituted for the wild-type (wt) cytoplasmic domain by subcloning into a dexamethasone (Dex)-inducible mammalian expression vector, pLK (Hirt et al., 1992) containing full-length E-cadherin.

Cell Culture, Transfection, and Immunofluorescence

All cell lines were grown in DME supplemented with 10% FCS, 1% L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO BRL). To generate stable cell lines, MDA231 or A431D cells were transfected with the pLK based vectors described above by standard calcium phosphate methods, and colonies selected in puromycin. Stably transfected cell lines were isolated with cloning cylinders and subsequently maintained in the absence of puromycin.

Immunofluorescent localization procedures have been described in detail (Reynolds et al., 1994). For most experiments, cells were fixed in 100% methanol at -20°C for 7 min. Primary antibodies were the following: hECD1 used at 0.1 $\mu\text{g/ml}$ (provided by Masatoshi Takeichi, Kyoto University, Kyoto, Japan); F1 α SH p120 polyclonal used at 0.1 $\mu\text{g/ml}$ (Wu et al., 1998); and anti- β -catenin polyclonal used at 1/5,000 (Sigma Chemical Co.). Secondary antibodies used were donkey anti-mouse Texas red, donkey anti-rabbit Cy2 (Jackson Labs) and donkey anti-rabbit Alexa 488 (Molecular Probes) each diluted 1/600. For actin staining, cells were fixed in 3% paraformaldehyde for 30 min at room temperature (RT), washed twice with PBS/10 mM glycine, permeabilized with PBS/0.2% Triton X-100 for 5 min at RT, and washed again with PBS glycine. Cells were otherwise stained as above except that actin was probed using Texas red-X phalloidin (Molecular Probes) at 1/600. Coverslips were mounted on glass slides with aqua Poly/Mount (Polysciences Inc.) and visualized under a Zeiss Axiophot fluorescent microscope. Photos were acquired with the IP Lab Spectrum program and compiled in Photoshop and Quark.

Immunoprecipitation and Western Blot Analysis

Procedures for immunoprecipitation and Western blotting have been described in detail (Reynolds et al., 1994). For coimmunoprecipitation experiments, cells were lysed in 0.5% NP-40 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, and 0.5% NP-40) containing protease inhibitors (1.5 mM PMSF, 1 mM EDTA, 0.1 mM sodium vanadate, 0.1 trypsin inhibitor units aprotinin, and 5 $\mu\text{g/ml}$ leupeptin; protease inhibitors are from Sigma Chemical Co.). For Western blotting of whole cell lysates, cells were directly lysed on tissue culture dishes with boiling 2 \times Laemmli sample buffer, briefly sonicated, and quantitated, before loading equal protein samples on SDS-PAGE gels. For Western blotting, mAb pp120 was used at 0.1 $\mu\text{g/ml}$, E-cadherin mAb hECD-1 was used at 0.1 $\mu\text{g/ml}$, plakoglobin mAb 15F11 was used at 0.25 $\mu\text{g/ml}$, and β -catenin (C2206; Sigma Chemical Co.) and α -catenin rabbit polyclonal antibodies (C-2081; Sigma Chemical Co.) were diluted 1/5,000. The vinculin ascites (V-4505; Sigma Chemical Co.) were diluted 1/1,000. Signals were detected using peroxidase-conjugated secondary reagents (D α M-HRP or protein A-HRP; Jackson ImmunoResearch Laboratories, both at 1/40,000) and enhanced chemiluminescence (Amersham).

Phosphatase Treatment

p120 was immunoprecipitated with 4 μg p120 mAb 15D2 from cells lysed in RIPA (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS) containing protease inhibitors (see above). Immunoprecipitates were subsequently washed three times with lysis buffer lacking EDTA and sodium vanadate, followed by two washes with TBS pH 7.4. Samples were split and incubated in the presence or absence of 200 U λ phosphatase (NEB 753S) in NEB λ phosphatase buffer for 30 min at 30°C . After removal of the incubation buffer, samples were processed for SDS-PAGE and Western blotting as described above.

Human Cadherin Juxtamembrane Domain Alignment

ClustalW alignment was performed using MacVector. GenBank accession numbers are as follows: E-cadherin, Z13008; N-cadherin, S42303; P-cadherin, X63629; R-cadherin (cadherin 4), L34059; VE-cadherin (cadherin 5), X79981; K-cadherin (cadherin 6), P55285; cadherin 8, D38992; cadherin 10, NM_006727; OB-cadherin (cadherin 11), L34056; cadherin 12, L34057; cadherin 14, U59325; cadherin 15, D83542; and cadherin 18, NP_004925.

Subcellular Fractionation

All procedures were done at 0°C . Adherent cells in 100-mm dishes were washed three times in PBS and allowed to swell for 15 min on the plate in 1.5 ml hypotonic buffer (10 mM Tris-HCl, pH 7.4, 1 mM MgCl_2) containing 1.5 mM PMSF, 1 mM EDTA, 0.1 mM sodium vanadate, 0.1 trypsin in-

hibitor units aprotinin, and 5 $\mu\text{g/ml}$ of leupeptin (Sigma Chemical Co.). Cells were scraped with a rubber scraper and broken by 10 strokes with a loose Dounce homogenizer, followed by 10 strokes with a tight Dounce. Cell breakage was monitored by phase-contrast microscopy until 90% of the cells were lysed with nuclei still intact. 5 M NaCl was added to a final concentration of 0.15 M. Nuclei were spun out by a 3-s spin at 14,000 rpm in a tabletop microcentrifuge and discarded. Cytosolic and membrane fractions were separated by ultracentrifugation for 30 min at 100,000 g at 4°C in a Beckman TLA100.3 rotor. The supernatant was removed and the pellet was washed twice with PBS. Both fractions were reconstituted to an equal volume containing a final concentration of 1 \times Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting.

Aggregation Assay

Cells were tested for their ability to aggregate in hanging drop suspension cultures. Cells were trypsinized in the presence of EDTA, washed twice in PBS, and resuspended with or without Dex at 5×10^5 cells per ml in DME. 1.5×10^4 cells in 30 μl of media were suspended as hanging drops from the lid of a 24-well culture dish and allowed to aggregate overnight in a humid 5% CO_2 incubator at 37°C . Corresponding wells were filled with PBS to prevent drying of the drops. Aggregation was assessed 18 h after plating. To assay for tightness of cell-cell adhesion, cells were subjected to shear force by passing them 10 times through a standard 200- μl gilson pipet tip. Cells were photographed within 20 min through a Zeiss Axiovert microscope 10 \times phase-contrast objective. For quantitation, individual fields of cells were counted after the pipetting stress, and the data was normalized according to the formula $(N_0 - N_i)/N_0$, where N_0 is the number of individual (nonaggregated) cells and N_i is the total number of cells in the sample.

Results

p120 Is Located in the Cytoplasm of Cells Lacking Functional Cadherins

E-cadherin and p120 colocalize at epithelial cell junctions. To test the hypothesis that p120 localizes to junctions primarily, if not solely, through its interaction with cadherins, we first examined p120 localization in carcinoma cell lines reported to lack E-cadherin. Fig. 1 compares by immunofluorescence the localization of p120 in the relatively normal E-cadherin-positive breast cell line, MCF7, and the E-cadherin-negative cell line, MDA-MB-231 (MDA231). Whereas MCF7 cells display normal E-cadherin and p120 staining at junctions (Fig. 1 A, i and ii), MDA231 cells lack E-cadherin (iv) and p120 was localized diffusely in the cytoplasm (iii). In some cells nuclear staining was evident (data not shown), as reported by others (van Hengel et al., 1999).

In several other E-cadherin-negative cell lines (Fig. 1 B), including A431D (i), CHO (ii), L-cells (iii), SKBr3, and BHK (data not shown), p120 was also diffusely localized, suggesting that in the absence of functional cadherins, p120 cannot associate with cell junctions. In some E-cadherin-negative cell lines such as HBL-100 and MDA 435, p120 was still associated with regions of cell-cell contact, but subsequent analysis revealed expression of N- or P-cadherin at those junctions (data not shown). Whereas parental A431 cells contain E- and P-cadherins, A431D cells do not express E-, N-, or P-cadherin, and lack reactivity to pan-cadherin antibodies (Lewis et al., 1997). Analyses of MDA231 cells by reverse transcriptase-PCR and Western blotting reveal a single weak cadherin band identified as cadherin 11 (Nieman et al., 1999; Pishvaian et al., 1999). The cadherin 11 in MDA231 apparently does not induce classical cadherin-like junctions, nor does it recruit

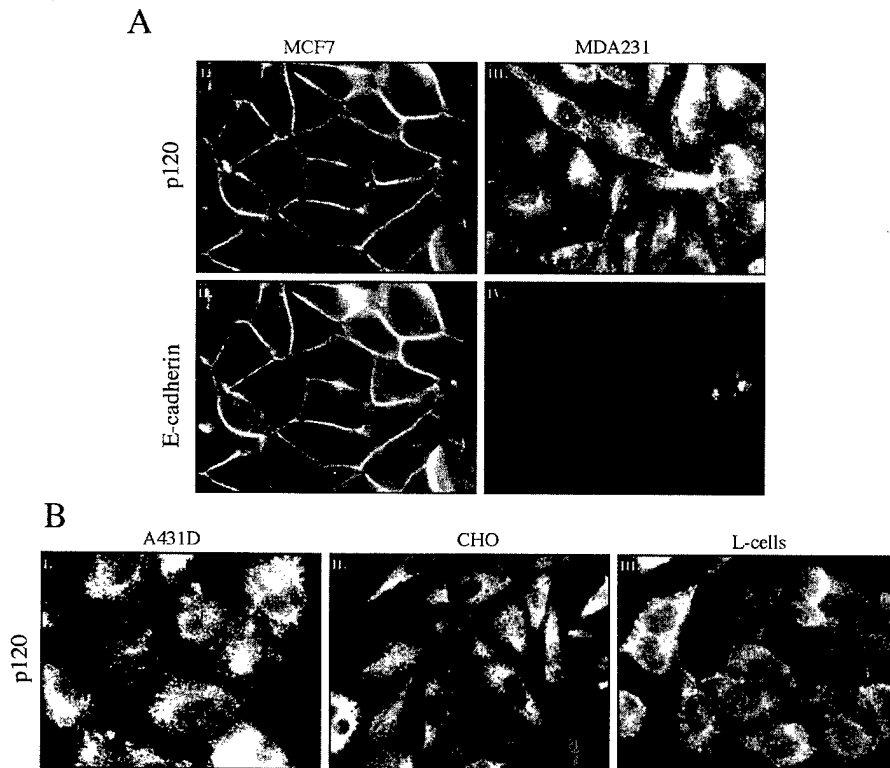


Figure 1. p120 mislocalizes to the cytoplasm in cadherin-deficient cells. (A) p120 (i and iii) and E-cadherin (ii and iv) were colocalized by immunofluorescence in a well differentiated human breast cell line MCF7 (i and ii) or the highly invasive E-cadherin-deficient breast cell line MDA231 (iii and iv). (B) Localization of p120 in the cadherin-deficient cell lines A431D (i), CHO (ii), and L-cells (iii). Note aberrant mislocalization of p120 to the cytoplasm in cadherin-deficient cells.

p120 to membranes (Fig. 1 A, iii). These results strongly suggest that the predominant localization of p120 at adherens junctions requires functional junction-forming cadherins, and that high levels of cytoplasmic p120 indicate the absence or malfunction of such cadherins. Of the cells with cytoplasmic p120, MDA231 and A431D cells were chosen as models for further analysis.

Recruitment of p120 to Cell Junctions by Ectopic Expression of E-cadherin

To confirm that mislocalization of p120 in E-cadherin-negative cells was due to the absence of E-cadherin, we stably transfected E-cadherin into MDA231 or A431D cells. To enable control of expression levels, we used the Dex-inducible pLK expression vector containing wild-type human E-cadherin cDNA. Stable E-cadherin-expressing cell lines were isolated and the effects of ectopic E-cadherin expression were examined by immunofluorescence in the presence of Dex.

In MDA231 cells, forced expression of E-cadherin caused a striking mesenchymal to epithelial transformation (Fig. 2, compare ii and iv). A431D cells also acquired tighter cell-cell junctions after E-cadherin expression (Fig. 2, compare vi and viii), but the morphological changes were less obvious, in part because these cells already resemble epithelia. In both cell lines, there was a striking recruitment of p120 from the cytoplasm to cell-cell junctions (compare ii to iv and vi to viii), where it colocalized with the ectopically expressed E-cadherin (iii and vii). α - and β -catenin were also recruited to junctions, where they colocalized with p120 and E-cadherin (data not shown). In addition to the junctional E-cadherin, all of the cell lines ectopically expressing E-cadherin exhibited variable

amounts of aberrantly localized E-cadherin in the region of the Golgi apparatus that probably reflects incompletely processed protein. These data indicate that E-cadherin expression is sufficient to recruit p120 to cell-cell junctions.

To further examine the idea that p120 exists at membranes solely because of interaction with cadherins, we localized p120 in A431D cells stably transfected with N-cadherin (Fig. 2, x) or P-cadherin (xii). Whereas the character of the junctions formed by each cadherin was noticeably different, both of these classical cadherins strongly recruited p120 to cell junctions, where the proteins precisely colocalized (Fig. 2, compare ix to x and xi to xii). These data strongly support the notion that cytoplasmic p120 staining reflects the loss of functional cadherins and/or the inability of cadherins to form junctions.

Cell Line Characterization and p120 Stability in the Absence of Cadherins

To characterize our stable cell lines and determine whether p120 expression or stability was affected by E-cadherin, individual E-cadherin-transfected cell lines were analyzed in the presence or absence of Dex by Western blotting. We first determined the effects of dose and time of Dex addition on E-cadherin expression and localization in the MDA231 cell lines. Clones 1 and 2 were tightly regulated by Dex and examined further. Whereas 10^{-6} M Dex is routinely used with cells expressing the pLK vector (Hirt et al., 1992), 10^{-8} M Dex was sufficient for nearly maximal E-cadherin expression (Fig. 3 A), and this concentration was used for all assays. Dex did not affect the morphology of the parental cells, nor were there obvious effects on the expression of any component of the cadherin complex (data not shown). E-cadherin expression

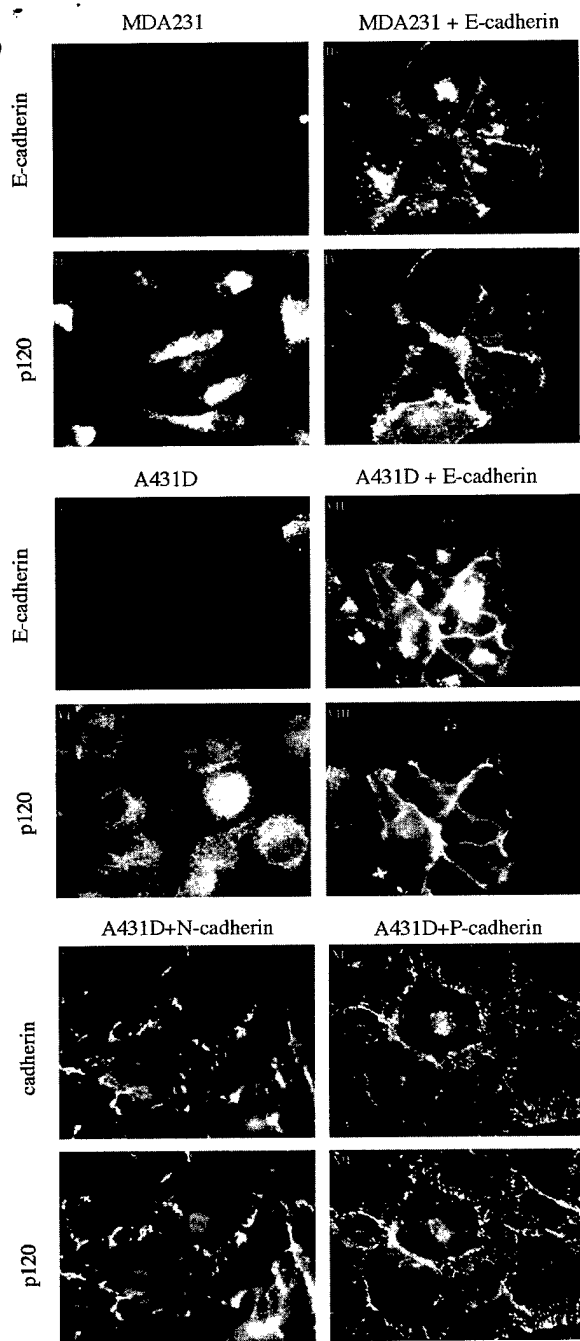


Figure 2. Cadherin expression is sufficient for recruitment of p120 to cell-cell junctions. p120 was localized by immunofluorescence in the cadherin-deficient cell lines MDA231 (i–iv) or A431D (v–viii) in the absence (i, ii, v, and vi) or presence (iii, iv, vii, and viii) of stably transfected E-cadherin. Restoring E-cadherin expression induces tight cell-cell junctions and the striking recruitment of p120 from the cytoplasm to cell-cell junctions. Stable ectopic expression of N-cadherin (ix and x) and P-cadherin (xi and xii) in A431D cells, as indicated, also induced junctional recruitment of p120 (x and xii) that colocalized with N-cadherin (ix) and P-cadherin (xi), respectively.

was first detected within 2 h after addition of Dex, and reached a maximum expression by 16 h (Fig. 3 B). Peak levels approximated the levels of endogenous E-cadherin present in control MCF7 cells (Fig. 3 C). Subsequent ex-

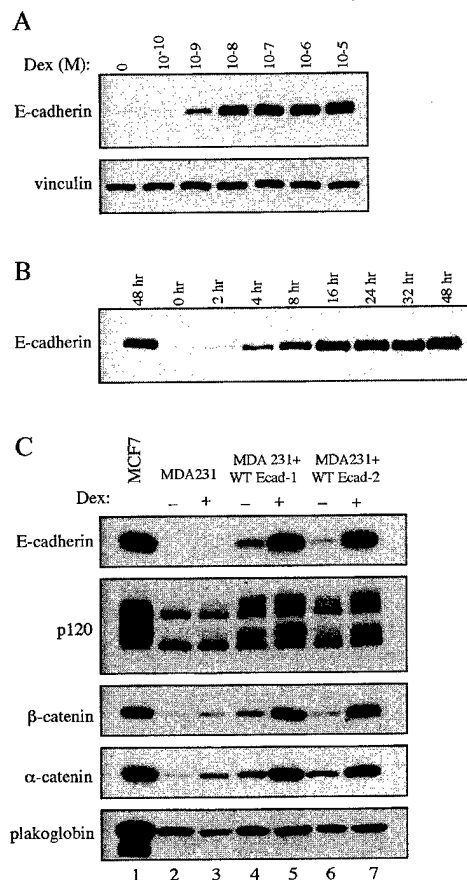


Figure 3. Biochemical status of the catenins in the absence and presence of ectopic E-cadherin. (A and B) Characterization of E-cadherin induction by Dex. Stable MDA231 cell lines expressing Dex-inducible E-cadherin were isolated. In A, cells were split equally and incubated 16 h in the presence of the indicated concentrations of Dex. Vinculin expression was monitored for loading and specific Dex induction of E-cadherin. In B, cells were split equally and incubated in 10^{-8} M Dex for increasing amounts of time, as indicated. In both experiments, whole cell lysates were collected and examined for quantitative expression of E-cadherin by Western blotting. The optimal condition for E-cadherin induction was treatment of cells with 10^{-8} M Dex for 16 h. Dex had no effect E-cadherin or catenin expression in untransfected wt MDA231 cells. (C) Untransfected (lanes 2 and 3) or E-cadherin-transfected (lanes 4–7) MDA231 cell lines were analyzed for the indicated proteins by Western blotting of whole cell lysates after incubation (as above) in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5, and 7) of Dex. In lane 1, the same proteins from the well differentiated E-cadherin-positive cell line MCF7 were analyzed on an equal protein basis to illustrate physiological levels of the respective cadherin complex proteins. Note the major band shift of p120 upon induction of E-cadherin.

periments were conducted after 16-h treatment with 10^{-8} M Dex.

Addition of Dex increased E-cadherin levels by ~5–20-fold depending on the cell line, correlating with dramatically increased levels of α - and β -catenins (Fig. 3 C, α - and β -catenin, compare lane 3 to lanes 5 and 7). In striking contrast, p120 was stable in the absence of cadherin expression (Fig. 3 C, p120, compare lanes 2 and 3 to lanes 4 and 5 or lanes 6 and 7). Although p120 levels appeared

marginally higher when E-cadherin was induced, the increased signal was due mostly to the major band shift that occurred because of p120 phosphorylation upon recruitment to cell junctions (see Fig. 9). Interestingly, unlike β -catenin, plakoglobin was expressed at moderate levels even in the absence of E-cadherin (Fig. 3 C, plakoglobin, lanes 2 and 3) and was not affected by ectopically expressed E-cadherin, indicating that its stability in these cells is regulated differently from that of β -catenin. These data show that the metabolic stability of p120 does not require interaction with cadherins, and suggest the absence of an APC-like mechanism to downregulate cytoplasmic levels of p120.

Fine Mapping of p120 Binding Site within the E-cadherin Juxtamembrane Domain

p120 and the p120 relative, δ -catenin/NPRAP, interact with the juxtamembrane domain (Lu et al., 1999; Yap et al., 1998) in agreement with yeast two-hybrid interaction assays performed in our lab and Mark Peifer's lab (University of North Carolina, Chapel Hill, North Carolina) (data not shown). However, experiments based on this data, have relied on the use of deletion mutants, which remove large segments of the cytoplasmic domain. To examine roles of the juxtamembrane domain that might be specifically attributed to p120, we first sought to identify minimal substitutions capable of selectively uncoupling the E-cadherin-p120 interaction. A series of three amino acid alanine substitutions was introduced within the juxtamembrane domain, and the constructs were assayed for interaction with p120 in the yeast two-hybrid system (Fig. 4). Fig. 4 A shows a schematic of E-cadherin, denoting the juxtamembrane domain and catenin binding regions. An amino acid alignment of the juxtamembrane domain from several human cadherins illustrates the very high conservation in this region. Fig. 4 B shows the outcome of yeast two-hybrid p120 interaction assays examining

the consequences of three amino acid substitutions (black boxes). p120 binding required the most highly conserved residues in the core of the conserved juxtamembrane domain, particularly those spanning amino acids D758-L773. This region is the most highly conserved sequence in the cadherin superfamily. Several of the juxtamembrane domain mutants failed to interact with p120, suggesting that the core region of the juxtamembrane domain is the most critical for p120 binding. Mutants 761AAA (GGG to AAA) and 764AAA (EED to AAA) were chosen for further study and subcloned into the pLK expression vector for analysis in the MDA231 and A431D model systems.

Uncoupling of p120 and E-cadherin in Mammalian Cells

The previous experiments implicate E-cadherin in the direct recruitment of p120 to junctions, but do not rule out the possibility of a secondary association of p120 with other junctional proteins that might accumulate in cadherin-induced cell-cell contacts. To test this possibility and confirm the yeast two-hybrid mapping data in mammalian cells, we generated stable A431D cell lines expressing the 761AAA and 764AAA mutants and MDA231 cell lines expressing the 761AAA mutant. For unknown reasons, we were unable to obtain stable 764AAA transfectants in MDA231 cells. Fig. 5 shows that p120 coimmunoprecipitated efficiently with E-cadherin from cells expressing wild-type E-cadherin (lane 5), but not at all from cells lacking E-cadherin (lane 2), or cells expressing 761AAA E-cadherin (lane 8). Similar results were obtained in A431D cells, where both the 761AAA and 764AAA E-cadherins failed to coprecipitate p120 (data not shown). β -Catenin coprecipitated with both wild-type and mutant E-cadherins (see Fig. 7), suggesting that conformations adopted by the mutant proteins do not significantly disturb other binding interactions of the cytoplasmic domain.

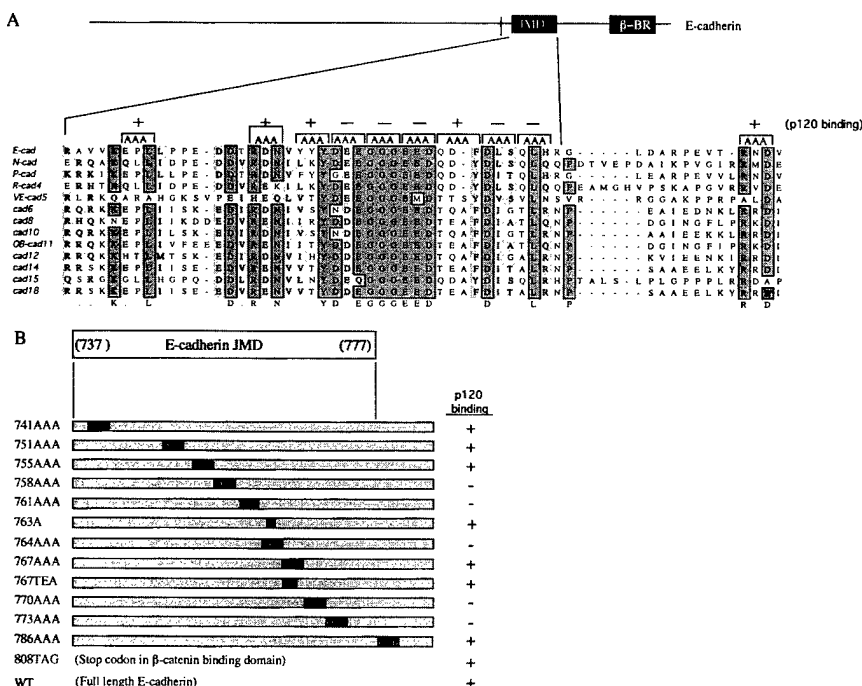


Figure 4. Fine mapping of the p120 binding site in the E-cadherin juxtamembrane domain. (A, top) Schematic representation of E-cadherin showing the scale the JMD, which binds p120, and the catenin binding region, which binds β -catenin. The sequence alignment below includes the region of the JMD that was analyzed by mutation. Triple alanine substitutions are indicated just above the alignments, and the results (+ or -) of yeast two-hybrid interaction assays for p120 binding to these mutants are shown. (B) Schematic depiction of the AAA substitutions made throughout the JMD of E-cadherin. The residues in black boxes were mutated to alanines, as indicated on the left. Binding of the respective mutants to p120 in yeast two-hybrid assays is shown on the right. The most highly conserved residues in the core of the JMD from D758 to L774 appear to be critical for p120 binding.

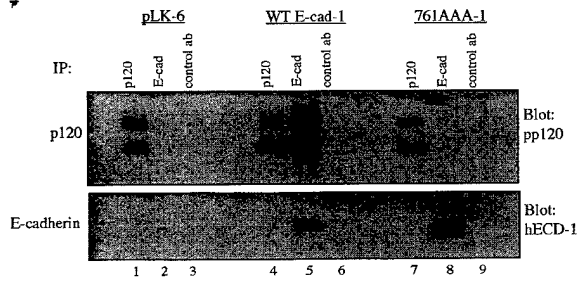


Figure 5. Failure of JMD mutant E-cadherins to associate with p120 in mammalian cells. NP-40 cell lysates from the indicated cell lines were immunoprecipitated with mAbs to p120 (lanes 1, 4, and 7), E-cadherin (lanes 2, 5, and 8), or the irrelevant control antibody 12CA5 (lanes 3, 6, and 9). The samples were Western blotted to detect coprecipitation of p120 (top), stripped, and reblotted for E-cadherin (bottom). Note, only 5% of the p120 direct immunoprecipitation samples (lanes 1, 4, and 7) was loaded so that long exposures could be used to analyze the less efficient coprecipitation of p120 with E-cadherin in the adjacent lanes. p120 coimmunoprecipitated efficiently with wt E-cadherin (lane 5), but not at all with the 761AAA JMD E-cadherin mutant (lane 8), or from cells expressing empty vector alone (pLK-6, lane 2).

In agreement with the coprecipitation data, ectopically expressed wild-type E-cadherin efficiently recruited p120 to junctions where the two proteins exactly colocalized (Fig. 6, i and ii), whereas both 761AAA (data not shown) and 764AAA E-cadherins localized to junctions, but failed to recruit p120, which remained stranded in the cytoplasm (Fig. 6, compare iii to iv). Whereas traces of p120 could sometimes be detected at 761AAA-induced junctions (data not shown), p120 was completely absent from 764AAA-induced junctions. Wild-type and mutant E-cadherins were indistinguishable in their respective abilities to stabilize and recruit β -catenin (Fig. 6, vi) and α -catenin (data not shown), illustrating the selective deficiency of p120 binding. When plated at high density, confluent cells expressing wild-type and mutant cadherins appeared to have similar if not identical cell contacts, showing that p120-uncoupled cadherins were not markedly deficient in establishing cell-cell contacts. The failure of p120 to asso-

ciate with these contacts shows definitively that the presence of functional cadherin is not only sufficient, but also necessary, for recruitment of p120 to cell-cell junctions. Interestingly, in subconfluent cultures, p120-uncoupled E-cadherins were significantly less efficient than wild-type cadherin in generating continuous and closely joined cell-cell contacts (Fig. 6, compare the spaces between cells in iii to the closely knit cells in i). These data strongly suggest that p120 is required for the consolidation of nascent contacts into strong and continuous cell-cell junctions.

Stoichiometry of p120 Association with Cadherins

In normal epithelial cells, p120 colocalizes precisely with E-cadherin, suggesting that the two proteins exist in a stoichiometric complex (Reynolds et al., 1994). However, previous studies suggest that very little (2–24%) of the p120 in cells is cadherin associated (Shibamoto et al., 1995; Stadon et al., 1995; Papkoff, 1997; Ozawa and Kemler, 1998). This idea stems in part from the inefficient coprecipitation of E-cadherin with p120, as compared with other catenins, in the presence of nonionic detergents. In the MDA231 and A431D systems, the ectopically expressed E-cadherin is the only cadherin expressed at cell junctions. Having established that p120 exists at the membrane solely because of cadherin expression, we employed a detergent-free subcellular fractionation assay to determine the stoichiometry of E-cadherin/p120 binding.

MDA231 cells stably expressing E-cadherin or the p120-uncoupled mutant 761AAA were biochemically fractionated by hypotonic lysis into cytoplasmic (C) and membrane (M) fractions, and assayed by Western blotting for the distribution of E-cadherin, p120, and β -catenin (Fig. 7). In vector alone transfected MDA231 cells, over 90% of the p120 fractionated to the cytosol (Fig. 7, compare lanes 1 and 2), consistent with the cytoplasmic staining observed by immunofluorescence. Ectopic expression of wild-type E-cadherin resulted in a dramatic shift of over 90% of the p120 into the membrane fraction (Fig. 7, compare lanes 3 and 4). By contrast, in the p120-uncoupled E-cadherin cell lines, p120 was found almost exclusively in the cytoplasmic fraction (Fig. 7, compare lanes 5 and 6) even though the

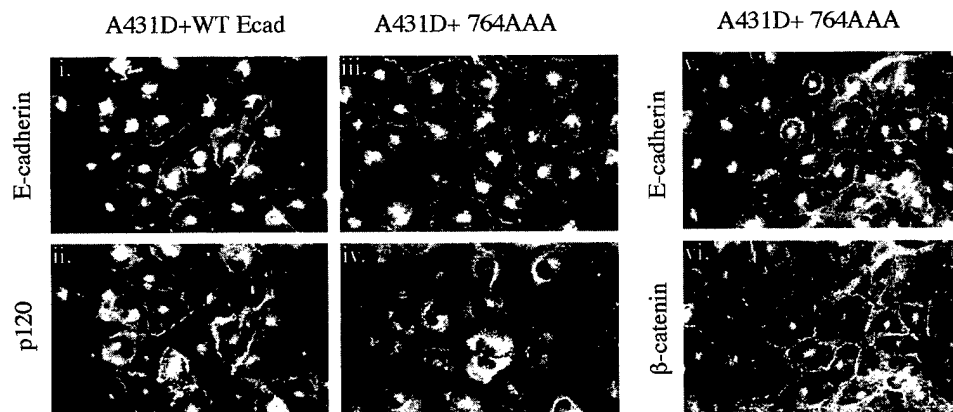


Figure 6. Association of p120 with E-cadherin is necessary for recruitment of p120 to junctions. wt E-cadherin (i and ii) or the JMD mutant 764AAA E-cadherin (iii–vi) were stably expressed in A431D cells and analyzed by coimmunofluorescence for colocalization of E-cadherin (i, iii, and v), with p120 (ii and iv), or β -catenin (vi). The JMD substitution mutant 764AAA E-cadherin localizes properly to junctions and colocalizes with β -catenin, but selectively fails to recruit p120. Formation of junctions by mutant E-cadherin is not sufficient for p120 recruitment. Instead, it is necessary that E-cadherin binds p120 directly.

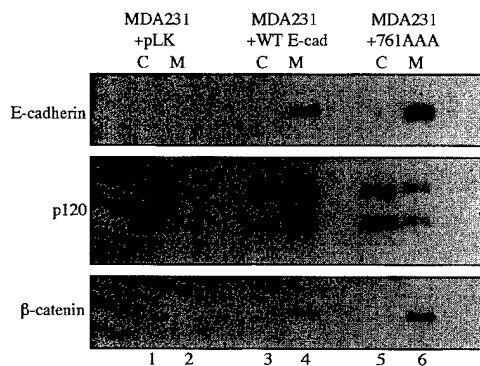


Figure 7. High stoichiometry of E-cadherin-p120 interaction. Having shown that E-cadherin is both necessary and sufficient for recruitment of p120 to the membranes, the stoichiometry of the interaction was estimated in the absence of detergents by hypotonic cell lysis and biochemical subcellular fractionation (see Materials and Methods). Control MDA231 cells (vector alone, MDA231 + pLK) were compared with the same cells expressing wt E-cadherin (MDA231 + WT Ecad), or the 761AAA substituted E-cadherin (MDA231 + 761AAA). The nuclear fraction was discarded, and cytoplasmic (C) and membrane (M) fractions from equal numbers of cells were analyzed by direct Western blotting with antibodies to E-cadherin, p120, or β -catenin (as indicated on the left) for quantitative comparison of association with membranes or cytoplasm. Approximately 90% of the p120 cofractionated with E-cadherin in cells expressing wt E-cadherin (compare lanes 3 and 4), whereas the opposite was true in E-cadherin-deficient cells (compare lanes 1 and 2), or in JMD mutated cells (compare lanes 5 and 6). p120, but not β -catenin, is stable in the absence of cadherin binding.

mutant cadherins and β -catenin (Fig. 7, lane 6) remained associated with membranes. Thus, wild-type E-cadherin expression caused the majority of the p120 to shift from the cytoplasm to the membrane, and this was completely dependent on the ability of the transfected E-cadherin to bind p120. We conclude that the vast majority of p120 in these cells is associated with E-cadherin, and in the absence of E-cadherin, p120 is stranded in the cytoplasm.

p120 Phosphorylation Requires Recruitment to the Membrane

In cadherin-deficient cells, p120 migrated on SDS gels as sharply focused bands. In contrast, ectopic E-cadherin expression in both MDA231 and A431D cells correlated with broad, diffuse p120 bands (Figs. 8 and 3 C), which resembled the p120 mobility shift observed by others (Ratcliffe et al., 1997; Aono et al., 1999; Ohkubo and Ozawa, 1999). Fig. 8 A shows the progressive upward band shift of p120 with time after the induction of E-cadherin by Dex addition. At time zero, both of the major p120 isoforms in these cells appear mostly dephosphorylated. The band shift to the upper form is complete by 16 h and correlates precisely with the induction profile of E-cadherin (Fig. 3 B). Moreover, p120-uncoupled cadherins did not promote phosphorylation of p120 as assessed by band shifts (Fig. 8 B, lanes 4–7). In contrast, wild-type E-cadherin clones (Fig. 8 B, lanes 2 and 3), or irrelevant point mutants (Fig. 8 B, lane 8) induced normal band shifts. These data indicate

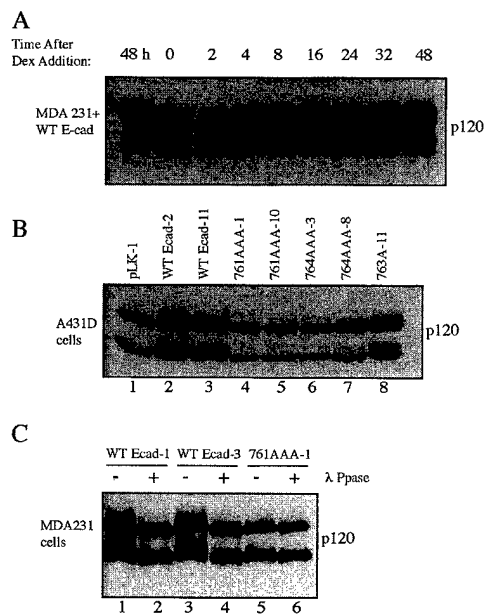


Figure 8. Recruitment to membranes by E-cadherin is prerequisite for p120 phosphorylation. (A) E-cadherin expression in MDA231 cells was induced by treatment with Dex for the indicated times, as in Fig. 3 B, and whole cell lysates analyzed by Western blotting for p120. A striking mobility shift of p120 is observed that correlates precisely with the induction kinetics of E-cadherin. (B) Cell lysates of A431D cells stably transfected with the indicated constructs were normalized for equal protein and Western blotted for p120. p120 mobility in cells expressing vector alone (pLK-1) was identical to that in cells expressing various p120-uncoupled E-cadherin mutants (lanes 4–7). In contrast, p120 was shifted up strongly in cell lines expressing wt E-cadherin (lanes 2 and 3) or the single alanine substitution mutant of E-cadherin (763A) that does not uncouple p120 (lane 8). (C) Phosphatase treatment (lanes 2, 4, and 6) of p120 immunocomplexes derived from MDA231 cells expressing wt E-cadherins (lanes 1–4) eliminate the band shift induced by recruitment of p120 to membranes (lanes 2 and 4) but has no effect on the cytoplasmic p120 found in cell expressing the p120-uncoupled E-cadherin 761AAA (compare lanes 5 and 6). Individual clonal cell lines are distinguished by numbers (e.g., 761AAA-1 and 761AAA-10).

that kinases acting on p120 are membrane associated, and suggest that recruitment of p120 to the membrane by cadherins is a prerequisite for subsequent activities triggered by these enzymes.

To confirm that the band shifts were due to phosphorylation, we analyzed the electrophoretic mobility of p120 before and after treatment with λ phosphatase, which acts on phosphorylated serine, threonine, and tyrosine. Phosphatase treatment collapsed the diffuse bands seen in the presence of wild-type E-cadherin into sharply focused bands (Fig. 8 C, compare lanes 3 to 4 and 5 to 6). This phosphorylation is likely almost entirely on serine, as we cannot detect tyrosine phosphorylation by sensitive anti-pTyr Western blotting of p120 from unstimulated A431D or MDA231 cells. Additionally, the bulk of p120 phosphorylation in cells is generally on serine (Reynolds, A., unpublished observation).

p120 Is Required for E-cadherin-mediated Transition from Loose to Tight Cell-Cell Adhesion

Although p120-uncoupled E-cadherin could largely restore the epithelial phenotype of MDA231 cells, the contacts between both MDA231 and A431D cells expressing these mutants were looser than those in cells expressing wild-type E-cadherin. Because organization of actin filaments can affect cell adhesion, we examined the cell lines for changes in the cytoskeleton. Actin staining revealed discontinuities in the actin compaction ring that circumscribes cell colonies, reflecting apparent defects in the attachment of actin cables to peripheral sites of cell-cell contact (Fig. 9 A). Normally, actin cables that circumscribe each cell split off near the cell perimeter to insert into concentrations of E-cadherin seen at the contact margin, a process that has been described in some detail in MDCK cells (Adams et al., 1998). This arrangement results in an almost continuous circumferential actin cable that is not confined to single cells, but instead binds entire colonies and likely accounts for the E-cadherin-mediated process referred to as compaction (for review see Adams and Nelson, 1998). In both the A431D (Fig. 9 A) and

MDA231 (data not shown) cells expressing p120-uncoupled E-cadherin, the circumferential actin cables failed to insert into peripheral concentrations of E-cadherin, resulting in circumferential actin rings that were confined to individual cells. In subconfluent cells, the actin defect resulted in colonies with irregular cell borders, as opposed to the tighter smooth border induced by wild-type E-cadherin (Fig. 9 A, compare iii and v). These observations suggest that the observed effects of p120 on adhesion may be a prerequisite for events associated with compaction, a phenomenon involving the organization and insertion of the actin cytoskeleton into sites of clustered E-cadherin.

To assay the effects of p120 uncoupling on cell-cell adhesion, we carried out aggregation assays designed to compare the strength of the adhesions mediated by wild-type or p120-uncoupled E-cadherin (Fig. 9 B). In the absence of E-cadherin, the A431D cells associated only loosely and separated immediately when passed through a pipet tip (ii). In contrast, wild-type E-cadherin induced tightly compacted cell aggregates that could not be dissociated by vigorous pipetting (iii) as was true for the original parental A431 cell line, which expresses E-cadherin (i). Cells ectop-

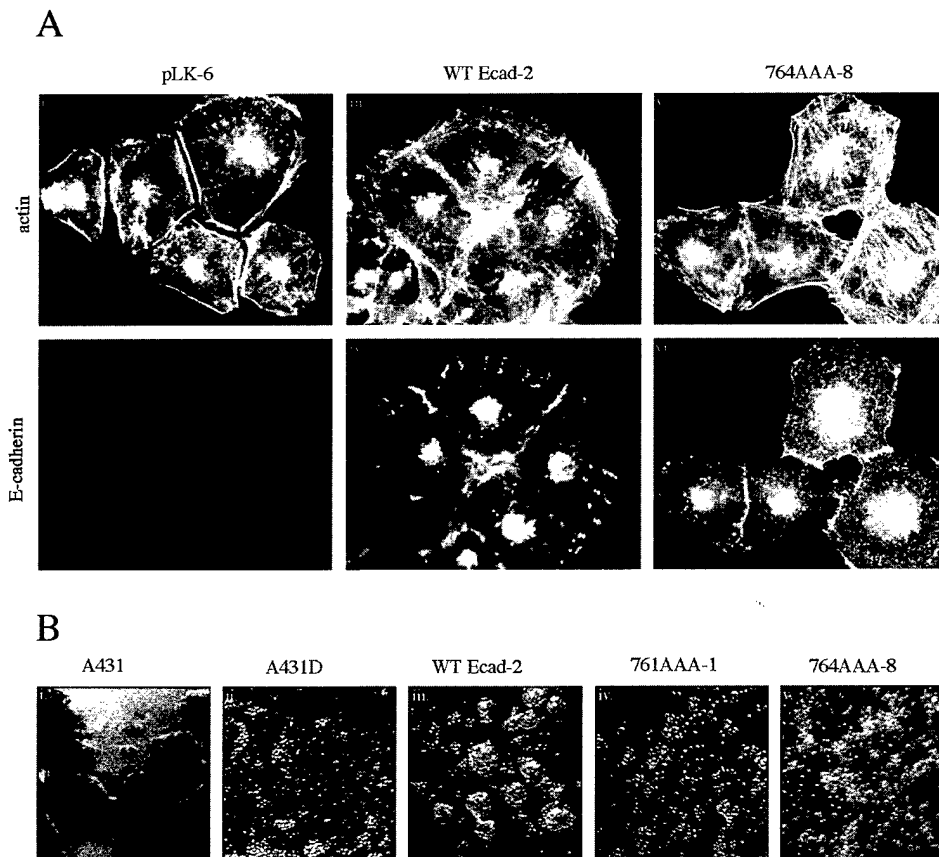


Figure 9. p120 is required for cytoskeletal organization and tight adhesion. (A) Defects in the actin cytoskeleton. A431D cells transfected with vector alone (pLK-6, i and ii), wt E-cadherin (WT Ecad-2, iii and iv) or the p120-uncoupled E-cadherin (764AAA-8, v and vi) were examined by immunofluorescence using the actin binding toxin phalloidin (conjugated to Texas red, i, iii, and v), and the E-cadherin Mab HECD1 (ii, iv, and vi). The relative absence of junctional connections in the cells lacking E-cadherin (pLK-6, i and ii) is readily evident. In cells expressing wt E-cadherin, concentrations of E-cadherin at peripheral cell contacts are evident (iv) and a continuous border of actin filaments circumscribes the entire colony (iii, arrows), connecting cells into a smoothly bordered colony by insertion into the cadherin plaques. In contrast, cells expressing the p120 uncouple mutant 764AAA fail to organize a cytoskeleton that efficiently connects adjacent cells. Instead actin filaments are disorganized (arrowheads) resulting in individual cells that organize poorly into colonies (v and vi). Actin bundles are not continuous from one cell to the next (v).

(B) Defects in adhesion. The aggregation characteristics of the cadherin-deficient A431D cells (ii) were compared with the same cells stably transfected with wt E-cadherin (iii), or p120-uncoupled cadherins 761AAA (iv) and 764AAA (v). The original parental A431 cell line, which is E-cadherin-positive, was also analyzed (i) for comparison. Cells were trypsinized, plated in equal numbers as hanging drop cultures, and allowed to aggregate overnight (18 h). Cells were dissociated by pipetting 10 times through a 200- μ l Gilson pipette tip. A431 cells (i) and wt E-cadherin expressing A431D cells (iii) formed compact aggregates that could not be dissociated by pipetting. A431D cells (ii) aggregated poorly and remained as single cells. In contrast, cells expressing p120-uncoupled cadherins (iv and v) aggregated better than the A431D cells, but dissociated immediately into single cells upon moderate pipetting.

ically expressing p120-uncoupled E-cadherin displayed tighter associations than the untransfected parental cells, but the cells could be visualized like individual grapes in a cluster, as opposed to the smooth tightly compacted aggregates induced by wild-type E-cadherin. The phenotype was obvious when the p120-uncoupled cell aggregates were passed through a pipet tip, which resulted in the rapid and complete dissociation into single cells (iv and v). Cell aggregation/disaggregation was quantitated by counting individual fields of these cells and using the formula $(N_0 - N_t)/N_0$ (see Materials and Methods), revealing the failure of pipetting shear stress to dissociate the parental A431 cells and A431D cells expressing wt E-cadherin (0.98 and 0.93, respectively). However, A431D-pLK cells, disaggregated readily (0.15), as did JMD-mutant cell lines 761AAA and 764AAA (0.14 and 0.20, respectively). These data suggest that the direct connection of E-cadherin to the actin cytoskeleton through β -catenin, α -catenin, and other factors such as α -actinin, are not sufficient for E-cadherin's ability to induce compaction. Instead, p120 association is crucial and regulates a key event necessary for the transition from loose to tight adhesion and potentially prerequisite for compaction. It is possible that the failure to recruit p120 reduces adhesion by inhibiting cadherin clustering, as has been proposed by Yap et al. (1998). Alternatively, p120 may somehow regulate the physical bridge to the actin cytoskeleton (consisting of β -catenin and α -catenin), or the organization of the actin cytoskeleton itself.

Discussion

We have uncoupled E-cadherin function from p120 by generating minimal substitutions in the E-cadherin juxtamembrane domain. By limiting the substitutions to three amino acids, we sought to minimize the risk of affecting non-p120 interactions, or alternatively, the possibility of unanticipated conformational effects. Indeed, these mutants have major advantages over the larger deletion mutants used in previous studies, and will be key reagents for pinpointing the role of p120 in the cadherin complex. A key observation from this work is that cadherins are both necessary and sufficient for the targeting of p120 to junctions. The data strongly suggest that cadherins are the only cellular proteins capable of recruiting p120 to membranes. The critical core residues found to mediate this binding, amino acids 758–773, constitute the most highly conserved region among members of the cadherin superfamily, suggesting a general indispensable role for the cadherin–p120 interaction. Indeed, we find that selective elimination of p120 from the cadherin complex severely reduces its ability to mediate the transition from loose to tight cell–cell adhesion.

In contrast to previous work, our data strongly suggests a stoichiometric interaction between cadherins and p120. It has been widely suggested that p120 is a relatively minor constituent of cadherin complexes (Shibamoto et al., 1995; Staddon et al., 1995; Papkoff, 1997; Ozawa and Kemler, 1998), with the highest published estimate of the amount of p120 binding to cadherins being 24% (Staddon et al., 1995). Indeed, only a fraction of p120 coprecipitates with cadherins in the presence of detergents, presumably be-

cause of a low affinity for cadherins in the presence of the nonionic detergents used to solubilize cells. In addition, the intensity of immunoblotted p120 is further diminished because p120 is expressed as multiple isoforms, each of which migrates diffusely on polyacrylamide gels because of extensive phosphorylation. Determining the stoichiometry of the interaction in some cells is further complicated by the existence of multiple cadherins. In our model of cell lines, p120 is stranded in the cytoplasm until E-cadherin is introduced by transfection. Using subcellular fractionation methods that do not require detergents, we have found that at least 90% of p120 in this simplified system cofractionates with E-cadherin. In cells expressing p120 uncoupled E-cadherin mutants, p120 is not recruited to the junctions formed by these mutant cadherins, demonstrating clearly that E-cadherin is the only protein in this system that can cause the membrane association of p120. These results likely underrepresent the true stoichiometry because our cell lines expressing wild-type E-cadherin are somewhat unstable, generally containing some cells (~5–10% of the total population) that have lost cadherin expression. The cytoplasmic p120 in these revertant cells would at least partially account for the appearance of a fraction of p120 in the cytosolic fraction. Thus, these biochemical results are in agreement with immunofluorescence colocalization data, where it is generally not possible to distinguish E-cadherin from p120 staining. Together, these data indicate that despite an apparently lower affinity, p120 is likely to be as abundant as β -catenin in cadherin complexes *in vivo*, and is unlikely under normal circumstances to exist as a significant unbound population.

Interestingly, the p120-uncoupled E-cadherin mutants were deficient in the reorganization of E-cadherin and actin that normally leads to the condensation of adjacent cells into compact epithelial colonies (Adams et al., 1998). The most obvious manifestation of this effect occurred in subconfluent cultures where groups of cells displayed uneven rather than smoothly compacted borders. Actin staining revealed a general absence of the peripheral actin cable that normally circumscribes the entire colony and results in a seamless connection between adjacent cells. Instead, each cell within a colony retained its own individual circumferential actin ring suggesting a lack of coordination with adjacent cells. Since these mutants associated with and stabilized α - and β -catenins, it is clear that the physical connection to the actin cytoskeleton mediated by these catenins is not sufficient by itself to drive compaction. Indeed, aggregation assays revealed that cells expressing p120 uncoupled E-cadherin were unable to form the tightly compacted cellular aggregates induced by wild-type E-cadherin. Instead, simple mechanical stress induced by moderate pipetting completely dissociated these cells, but had no effect on the tightly connected aggregates induced by wild type E-cadherin. Therefore, we postulate that p120 is necessary for the consolidation of loose adhesion, mediated in part by homotypic association of the extracellular domains, into tight adhesion mediated by additional rearrangement and attachment of the underlying actin cytoskeleton. Because p120 does not directly associate with α -catenin, it is likely that it binds other proteins which in turn act to regulate and remodel the actin–cadherin interface. The unusual ability of overexpressed p120 to induce

striking dendrite-like projections in fibroblasts (Reynolds et al., 1996) probably reflects an as yet unidentified activity that is required under normal circumstances for organization of the actin cytoskeleton in the context of the cadherin complex.

Recent publications reveal apparently conflicting roles for the juxtamembrane domain and presumably p120. Different lines of evidence suggest crucial roles in cell-cell adhesion (Kintner, 1992; Yap et al., 1998), cell motility (Chen et al., 1997), neuronal outgrowth (Riehl et al., 1996), and exclusion of one cadherin by another from adhesive contacts (Navarro et al., 1998). Because p120 binds to many cadherins, including E-, N-, P-, VE-, and C-cadherin (Reynolds et al., 1996; Lampugnani et al., 1997; Yap et al., 1998), some variation in the role of p120 might be expected because of interaction with different cell-type-specific cadherins. On the other hand, Yap et al. (1998) show that the juxtamembrane domain supports cadherin clustering and promotes strong cell-cell adhesion, whereas others including Aono et al. (1999), and Ohkubo and Ozawa (1999) suggest that p120 can negatively regulate adhesion. These apparent discrepancies are further complicated by the cell-type-specific expression of different p120 isoforms and increasing evidence that multiple signaling events regulate p120 activity, presumably through tyrosine and serine/threonine-directed phosphorylation (Reynolds et al., 1989; Downing and Reynolds, 1991; Ratcliffe et al., 1997; Aono et al., 1999; Ohkubo and Ozawa, 1999). Indeed, the negative effects of p120 on cadherin-mediated adhesion in Colo205 cells may reflect a special situation whereby constitutive signaling events, presumably leading to p120 phosphorylation, result in a constitutive role for p120 in dismantling cadherin complexes. In our cell lines, p120 was extensively phosphorylated on serine without obvious negative effects on cadherin-mediated adhesion. Thus, activation of p120's negative regulatory activity, as suggested previously (Aono et al., 1999), may be dependent on the selective phosphorylation of a specific site. Differences between our data and those of Ohkubo and Ozawa (1999) are more difficult to reconcile but may reflect inherent differences between cell types. Though we cannot completely rule out the possibility that proteins other than p120 are affected by our mutations, these relatively small substitutions are significantly less likely than deletions to affect other protein interactions in the complex. In general, the data suggest that p120 binding is required for E-cadherin to induce tight cell-cell adhesion. However, because we used cadherin mutants that did not associate with p120, we did not address the possibility that in the context of the cadherin complex, p120 might also negatively affect adhesion.

All of the cadherin-negative cell lines examined displayed a striking difference between the stability of p120 and β -catenin. As has been reported previously in other cell lines, α - and β -catenins were rapidly degraded in the absence of cadherin (Nagafuchi et al., 1991; Papkoff, 1997). In fact, α - and β -catenins were difficult to detect under these conditions by either Western blotting or immunofluorescence. Exceptions to this phenomena have been observed only in certain carcinoma cell lines, such as SW480 colon cells, where β -catenin is apparently stabilized in the absence of cadherin binding, because of a loss-

of-function mutation in APC or stabilizing mutations in β -catenin (Morin et al., 1997). In contrast, p120 was metabolically stable regardless of cadherin status, and easily detected at high levels in the cytoplasm. Ectopic expression of E-cadherin rescued the stability of α - and β -catenins, but had little effect on the levels of p120. Instead, ectopic E-cadherin recruited p120 from the cytoplasm to the membrane and coincided with extensive p120 phosphorylation as evidenced by a significant phosphatase-sensitive band shift on polyacrylamide gels. Indeed, cytoplasmic p120 was almost completely unphosphorylated, whereas at least 50% of the E-cadherin-associated p120 shifted in mobility. In contrast to β -catenin, plakoglobin levels were not at all affected by changes in cadherin expression. These data contrast with experiments in L-cells where plakoglobin is not present and changes in p120 phosphorylation were not observed after ectopic expression of cadherins (Papkoff, 1997). In addition, β -catenin association appears to be important for the correct targeting of E-cadherin to basolateral membranes (Chen et al., 1999). In contrast, our p120-uncoupled E-cadherin mutants localized properly to basolateral membranes in the absence of p120, indicating that p120 binding is not required for translocation and targeting of cadherins to regions of cell-cell contact.

The absence of p120 phosphorylation in the cadherin negative cells indicates that kinases impacting on p120 function are likely to exist at the plasma membrane. Interestingly, p120 was initially identified as a potent target of the Src tyrosine kinase (Reynolds et al., 1989). Src is normally tightly associated with membranes but can be rendered cytoplasmic by mutation of its NH_2 -terminal myristylation signal (Buss et al., 1984; Cross et al., 1984; Buss and Sefton, 1985; Kamps et al., 1985), and such mutants do not phosphorylate p120 (Reynolds et al., 1989). Apparently, the opposite scenario is also true; i.e., rendering p120 cytoplasmic by removing its cadherin anchor to the membrane eliminates most, if not all, of the otherwise extensive phosphorylation. However, Src is not overexpressed in MDA231 or A431D cell lines and the predominant phosphorylation observed in these cells was on serine. As mentioned previously, there is now significant evidence suggesting that serine phosphorylation of p120 may contribute to its ability to negatively regulate the adhesive function of E-cadherin (Aono et al., 1999; Ohkubo and Ozawa, 1999) and several cellular treatments that eliminate the p120 band shift (e.g., staurosporine) also restore functional adhesion properties to the cadherin complex. It would appear that anchoring p120 to a cadherin is a prerequisite for additional regulation through a variety of kinases, both tyrosine and serine/threonine-directed, which modify its activity further.

Our data also highlights a second possibility relating to cadherin loss, that the pleiotropic effects of cadherin loss in metastatic cells are due not only to defects in adhesion, but also to signaling contributed by the high levels of cytoplasmic p120. In the case of β -catenin, APC keeps cytoplasmic levels of β -catenin tightly in check, and the consequences of APC mutation or stabilization of β -catenin by direct mutation have dire consequences contributing to the initiation of colon cancer and melanoma (for review see Polakis, 1999). Clearly, p120 is not regulated by these

mechanisms. We have shown previously that p120 does not interact with APC (Daniel and Reynolds, 1995), and we show here that it is not degraded under circumstances that rapidly remove β -catenin. The question is whether this aberrant cytoplasmic population contributes to the malignant properties of cadherin negative cells. Several reports showing p120 absence, downregulation, or mislocalization in human tumors imply that dysregulation of p120 could contribute to the malignant phenotype (Dillon et al., 1998; Gold et al., 1998; Shimazui et al., 1996; Skoudy et al., 1996; Valizadeh et al., 1997; Syrigos et al., 1998). Thus, one interpretation of our data is that loss of p120 function would decrease cell-cell adhesion, potentially supporting increased cell motility and metastasis. However, in cells where cadherin expression is lost, high levels of cytoplasmic p120 might lead to increased nuclear translocation and activation or inhibition of additional signaling events. Two recent reports are consistent with a role for p120 in transcription. First, nuclear localization of p120 has been reported to be particularly evident in cadherin-deficient cells, including the MDA231 cells used in this study (van Hengel et al., 1999). We have also noticed this effect in our cell lines under some circumstances. In addition, we have identified a novel nuclear transcription factor, Kaiso, which interacts directly with p120, suggesting that p120 may regulate transcriptional activity at some level (Daniel and Reynolds, 1999). Thus, translocation of p120 to the nucleus and subsequent modification of transcriptional events are potential secondary consequences of cadherin loss. It is also possible that a critical role of E-cadherin is to regulate access of p120 to the nucleus by sequestering it at the cell membrane. Such a function could be crucial given the apparent absence of any mechanism to downregulate or degrade cytoplasmic p120 when cadherin expression is lost.

In summary, our experiments reveal that p120 is required for E-cadherin-induced compaction. The α - and β -catenin linkage by itself is clearly insufficient, and instead p120 appears to be needed to mediate the consolidation or insertion of the actin cytoskeleton at the E-cadherin-cytoskeleton interface. The cadherin mutants used in this study (containing minimal alanine substitutions) significantly reduce the chances that the observed effects are due to conformational artifacts or disruption of other cadherin-protein interactions and will be crucial reagents to further dissect the role of p120. The stoichiometry of the p120-cadherin interaction is much higher than previously reported, and indeed cadherins are both necessary and sufficient to recruit p120 to the membrane. This suggests that regulation of cadherin function is a predominant rather than secondary role for p120. Nonetheless, the data also suggest that E-cadherin might regulate an alternative function for p120 in the cytoplasm and/or nucleus by sequestering it at cell-cell junctions.

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